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Mechanistic studies on the phosphoramidite coupling reaction in oligonucleotide synthesis. I. Evidence for nucleophilic catalysis by tetrazole and rate variations with the phosphorus substituents

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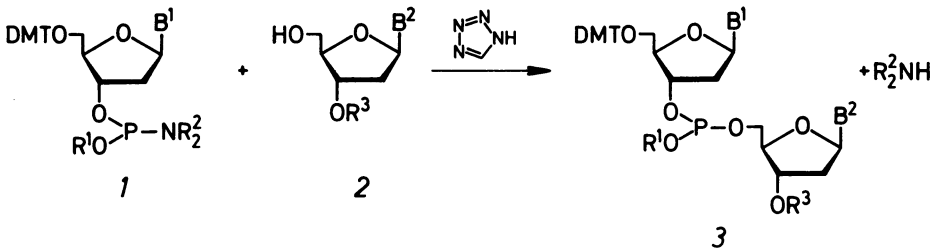
ABSTRACT

Tetrazole catalyzed reactions of a series of phosphoramidites, 5'-O-DMTdT-3'-O-P(OR^1)NR^2_2 (1a-h), with 3'-O-SiBu^tPh_2-6-N-benzoyl-dA (2a) in acetonitrile solution have been studied. It is found that the coupling rate depends very much on whether tetrazole is added before or after 2a, and that dialkylammonium tetrazolide salts are inhibitors. These and other facts are evidence that the reactions are subjected to nucleophilic catalysis by tetrazole, in addition to acid catalysis. The rate variations with phosphorus substituents of 1a-h are NEt_2 > NPr^i_2 > $N(CH_2CH_2)O$ > NMePh, and OMe > OCH_2CH_2CN > $OCHMeCH_2CN$ > $OCMe_2CH_2CN$ >> OC_6H_4Cl. The inhibitor properties of dialkylammonium tetrazolides have practical consequences for the efficiency of DNA syntheses, when *in situ* prepared phosphoramidites are used; the same would apply for segmented, simultaneous syntheses or syntheses where recycling is performed.

INTRODUCTION

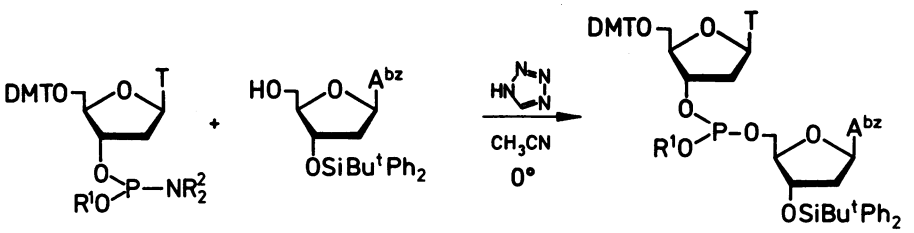
Deoxyribonucleoside phosphoramidites (1) are today the most widely used monomers for polymer-supported synthesis of DNA fragments.[1] The key step in the synthesis is the tetrazole catalyzed coupling reaction of 1 with a 5'-hydroxy group on the growing oligonucleotide chain (2, R^3 = polymer-bound oligonucleotide). A successful synthesis depends, *inter alia*, on this coupling reaction being fast and virtually quantitative and, therefore 1 should be highly reactive when activated with tetrazole. On the other hand it is desirable that 1 is unreactive in the absence of catalysts in order to facilitate purification and storage. The phosphoramidites originally introdúced by Beaucage and Caruthers (1, R^1 = R^2 = Me)[2] were highly reactive but difficult to purify and not very stable in solution. A higher stability was attained by replacement of the dimethylamino group of 1 with diisopropylamino[3,4] or morpholino[4,5] groups. The methoxy group of 1 has



likewise been replaced with other substituents, mainly in order to allow facile removal by β-elimination after DNA synthesis (1, R^1 = CH_2CH_2CN,[6] $CH_2CH_2SO_2R$,[7] or $CH_2CH_2C_6H_4NO_2$[8]), but also for stability or selectivity reasons (1, R^1 = CMe_2CH_2CN,[9] or C_6H_4Cl[10]).

Stability in this context means lack of reactivity of 1 towards nucleophiles (mainly water) in the absence of catalysts. The question arises whether the more stable phosphoramidites (1, NR^2_2 = NPr^i_2 or $N(CH_2CH_2)_2O$) are as reactive in the presence of tetrazole as 1 (R^2 = Me), or some sacrifice has been made in order to obtain the higher stability. It would also be of interest to know the relative reactivities of a larger range of nucleoside phosphoramidites under coupling conditions. No quantitative data have been published on this, and furthermore the mechanism of substitution, as well as the exact function of the catalyst, is largely unknown. For substitution reactions at a trivalent phosphorus center a mechanism *via* phosphoranes has been proposed,[11] although the inversion stereochemistry found in most cases points to a direct displacement (S_N2 type) mechanism.[12] It is generally accepted that all substitution reactions on phosphoramidites are acid catalyzed, *e.g.* by $R_2NH^+_2$ X^-,[2,13] but the proposed catalysis mechanism differs; mechanisms involving N-protonation,[14,15] P-protonation,[16,17] and/or nucleophilic catalysis by X^-,[14,18] have been postulated.

A knowledge of the mechanism is helpful to select the "best" phosphoramidite and catalyst among the many compounds proposed in the literature. We have therefore begun a study of the mechanism of the coupling reaction and present here our initial results in this respect: The function of the most commonly used catalyst, tetrazole, and the relative rates of coupling of a series of



1a–h 2a 3

1	a	b	c	d	e	f	g	h
R^1	Me	Me	Me	Me	CH_2CH_2CN	$CHCH_2CN$ (Me)	$C-CH_2CN$ (Me, Me)	Cl
NR^2_2	NEt_2	NPr^i_2	N O	NMePh	NPr^i_2	NPr^i_2	NPr^i_2	NPr^i_2

Scheme 1

phosphoramidites, 1a-h, with a suitably substituted nucleoside, 2a, in acetonitrile, catalyzed by tetrazole (Scheme 1).

RESULTS AND DISCUSSION

Eight 5'-O-dimethoxytritylthymidine-3'-O-phosphoramidites (1a-h) were selected to show the variation in rate with change of the N-substituents (1a-d) or the O-substituents (1b,e-h). The phosphoramidite (1, R^1 = R^2 = Me) originally proposed by Beaucage and Caruthers was not included because it could not be purified by column chromatography. The nucleoside 2a was selected for solubility reasons; most 3'-O,N-protected nucleosides are only sparingly soluble in acetonitrile at 0°C.

Several attempts were made to monitor the coupling reactions by ^{31}P NMR spectroscopy, but the sensitivity was too low to give satisfactory results. The reactions were instead followed by quenching samples in excess triethylamine and analyzing their composition after TLC separation by measuring the absorbance at 498 nm due to DMT^+ liberated from 1 and 3 by acid treatment.[19] The most serious problem was to avoid excessive hydrolysis during the reactions. By scrupulous drying of reagents, solvents and equipment (see Experimental for details) the hydrolysis of 1a-h

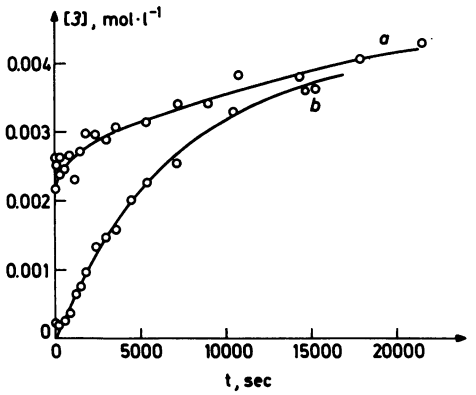


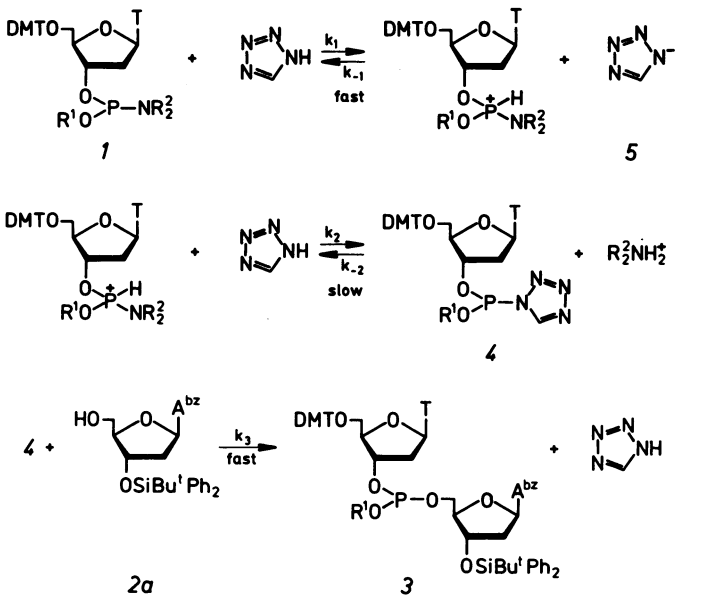
Fig. 1. Tetrazole catalyzed formation of phosphite 3 from phosphoramidite 1e and 2a *versus* time. a: 2a last added; b: tetrazole last added. C^{o}(1e) = C^{o}(tet) = 0.014 M, C^{o}(2a) = 0.007 M, solvent CH_3CN, 0°C.

was kept at acceptable level, in most cases less than 10% during the accumulation of data.

Tetrazole catalysis

The first experiments were performed by premixing 1 and tetrazole and adding 2a at the start of the measurements. However, the initial rates were very high and nearly independent of the N-substituents (Fig. 1, curve a). When 1 and 2a were premixed instead, and tetrazole added at time zero, a more gradual formation of product was observed (Fig. 1, curve b). The different results, when the order of addition of 2a and tetrazole was reversed, is interpreted in the following way. An equilibrium between 1 and a reactive intermediate, presumably 4 (Scheme 2), is established when 1 and tetrazole are premixed. The high rates observed initially in such experiments are the rates of reaction of 4 with 2a to give 3. The much lower rates seen after some time when 1 and tetrazole are premixed, and the rates observed when 1 and 2a are premixed, are the rates of formation of 4.

The mechanism of catalysis depicted in Scheme 2, *i.e.* acid catalysis *and* nucleophilic catalysis by tetrazole, is corroborated by several additional facts. The proposed intermediate 4 (R^1 = Me) has been prepared and shown to react very fast with alcohols.[20] The ^{31}P NMR chemical shift of 4 (R^1 = Me), 126 ppm in



Scheme 2

CH_3CN, is close to those of signals often observed by us and others[4,21] in mixtures of 1 and tetrazole. The signal intensity varies with the nature of substituents on phosphorus in 1 and increases with increasing amounts of tetrazole, in agreement with the establishment of an equilibrium between 1 and 4 as shown in Scheme 2. Further evidence for the mechanism of Scheme 2 is that addition of tetrazolide ions (5) significantly reduces the rate of formation of 3. An example is shown in Fig. 2, which shows that the rate is very retarded even with a 1:5 molar ratio of diisopropylammonium tetrazolide to tetrazole. This unexpected retardation is evidence for the proposed reversible and fast acid catalysis step, since the nucleophilic catalysis step being slow cannot account for the observed inhibition by diisopropylammonium tetrazole. Another evidence for nucleophilic catalysis and not solely acid catalysis comes from experiments with polymer-supported phosphoramidites described by Seliger.[22] He showed that phosphoramidites anchored to a solid support *via* a P-amino substituent on treatment with an acetonitrile solution of tetrazole

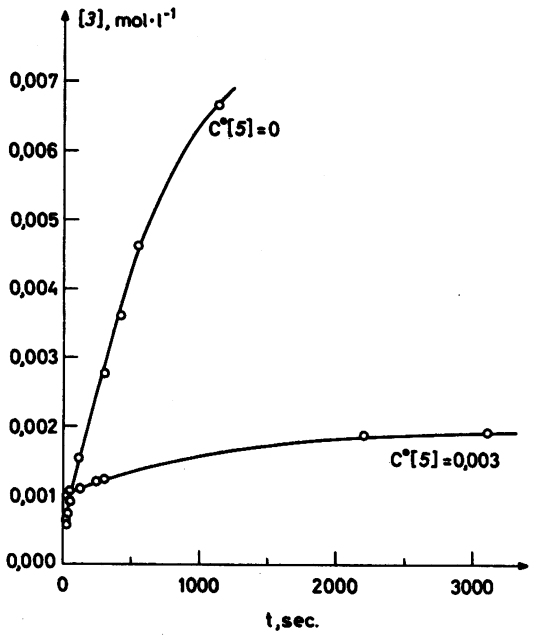


Fig. 2. Tetrazole catalyzed formation of phosphite 3 from phosphoramidite 1b and 2a *versus* time, with and without added diisopropylammonium tetrazolide 5. C^{o}(1b) = 0.030 M, C^{o}(2a) = C^{o}(tet) = 0.015 M, solvent CH_3CN, 0°C.

gave solutions containing active coupling reagents. These cannot be protonated 1, which would remain on the support, but are most probably the tetrazolides 4.

The effect of added salt (5) on the coupling rate has several practical consequences for efficient DNA synthesis using phosphoramidites. One is that phosphoramidites prepared *in situ* from nucleosides and alkyl phosphorodiamidites often contain 5[21,23] and therefore couple less efficiently than purified phosphoramidites. This, however, can be remedied by using a larger than usual excess of tetrazole during the couplings.[23] Another is relevant for segmented, simultaneous synthesis of oligonucleotides.[24,25] The amount of 5 formed during coupling (or hydrolysis) in each segment increases through the series of segments and may lead to insufficient coupling in the later segments. A similar build-up of 5 during recycling may explain why recycling is not

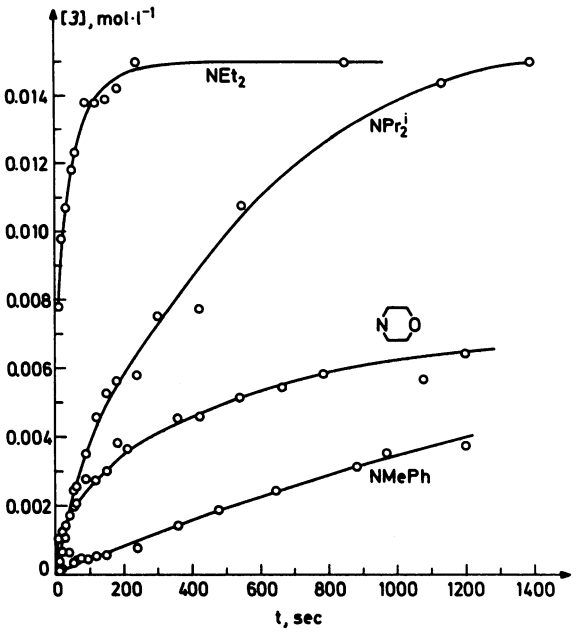


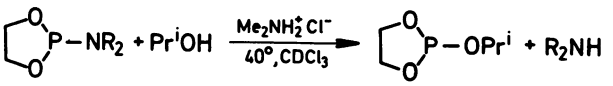
Fig. 3. Tetrazole catalyzed formation of phosphite 3 from methyl phosphoramidites 1a-d and 2a *versus* time. $C^{o}(1) = C^{o}(tet) = 0.030$ M, $C^{o}(2a) = 0.015$ M, solvent CH_3CN, 0°C.

very effective when phosphoramidites are used, although it is effective with phosphorochloridites.

Rate variations with phosphorus substituents

The complicated rate curves found when 1 and tetrazole were premixed (Fig. 1, a) lead us to abandon this approach for the present study, although the compounds are mixed in this way when DNA syntheses are performed. Thus the experiments described below are all made by premixing 1 and 2a and adding tetrazole at time zero. The results are presented as rate curves (conc. of 3 *versus* time) and not rate constants, since the data precision was considered too low to justify a numerical analysis.

The variation in rate with change of the N-substituents was first investigated (Fig. 3). The four reactions, starting from one of the phosphoramidites 1a-d, were run under identical conditions, and it is seen that the rate decreases through the series NEt_2 > NPr^i_2 > $N(CH_2CH_2)O$ > NMePh. Apart from NPr^i_2, which



(eq.1)

$$NPh_2 > NMePh > NMe_2 > NPr^i_2$$

probably makes 1b less reactive due to steric hindrance, the reactivity decreases with a decrease in the base strength of the corresponding amines in water (pK_a 10.49 for $Et_2NH_2^+$, 10.96 for $Pr^i_2NH_2^+$, 8.33 for $O(CH_2CH_2)_2NH_2^+$, and 4.85 for $PhMeNH_2^+$ [26]). This is probably opposite to the leaving group ability of NR_2 and opposite to the order of reactivity found in earlier model experiments (eq. 1).[17] However, the conditions of the two sets of experiments are very different, in particular the nature and amount of catalyst used. In the model experiments, the catalyst contained a poor nucleophile, Cl^-, and the catalyst concentration was low (2 mole-%); in the present experiments tetrazole is a good nucleophile, and the catalyst concentration is high (100 mole-%). Our working hypothesis is that the mechanism varies for the two cases. With small amounts of acidic catalysts and no good nucleophile present the mechanism is probably direct displacement (S_N2) on a protonated phosphorus atom; this fits the observed stereochemistry of predominant inversion[12] and the observed rate increase when NR_2 becomes a better leaving group. With substantial amounts of tetrazole as the catalyst the mechanism includes nucleophilic catalysis as depicted in Scheme 2. This explains the observed lack of stereoselectivity,[27] since the intermediate 4 is expected to epimerize fast at phosphorus by reaction with tetrazole. It may also explain why the rate increases with increasing basicity of R_2NH. In the presence of large amounts of acidic catalysts additional N-protonation probably occurs in the transition state, and this protonation is more developed for the more basic NR_2 groups.

The unexpected results of the rate variations with the N-substituents thus may be explained by the postulated mechanism. The results also constitute a warning only to use model reactions which closely simulate the reactions in question.

The variation in rate with change of the O-substituents is

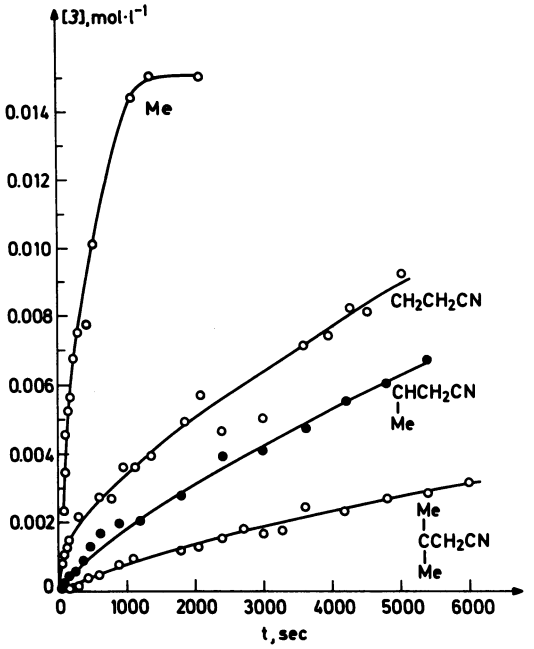


Fig. 4. Tetrazole catalyzed formation of phosphite 3 from alkyl diisopropylphosphoramidites 1b,e-g and 2a *versus* time. $C^o(1)$ = $C^o(tet)$ = 0.030 M, $C^o(2a)$ = 0.015 M, solvent CH_3CN, 0°C.

shown in Fig. 4. The rate is seen to decrease through the series Me > CH_2CH_2CN > $CHMeCH_2CN$ > CMe_2CH_2CN >> C_6H_4Cl. The 2-chlorophenyl group causes a very low reactivity of 1h (the rate is too low to measure by the technique used; ^{31}P NMR experiments showed that 1h reacts ca. 1000 times more slowly than 1e). A low reactivity is expected from resonance and inductive effects of an aryl group, however the effect is unexpectedly large. The 2-cyanoethyl group causes a minor rate reduction compared to methyl (ca. 4-fold), as expected from steric reasons and from inductive effects of the cyano group. A 2-3-fold decrease in rate is found going from CH_2CH_2CN to $CHMeCH_2CN$ and from $CHMeCH_2CN$ to CMe_2CH_2CN, *i.e.* the reaction shows a modest sensitivity to steric hindrance.

CONCLUSIONS

The data presented here are the first published quantitative data related to the reactivity of different phosphoramidites 1

under conditions close to those used for DNA synthesis. It is found that the measured rate depends very much on whether tetrazole is added before or after the hydroxy compound 2a. This is explained by a mechanism involving nucleophilic catalysis as well as acid catalysis by tetrazole (Scheme 2). The proposed mechanism explains the lack of stereoselectivity and the inhibitor properties of ammonium tetrazolides. The rate variations with the N-substituents, NEt_2 > NPr^i_2 > $N(CH_2CH_2)_2O$ > NMePh, which is different from earlier results from model experiments, are also explained by the mechanism. The variation with the O-substituents is as expected from inductive and steric effects, Me > CH_2CH_2CN > $CHMeCH_2CN$ > CMe_2CH_2CN >> C_6H_4Cl.

The results have several practical consequences for efficient DNA synthesis. One is the inhibitor properties of ammonium tetrazolides, which is important when *in situ* prepared phosphoramidites are used, and when segmented, simultaneous synthesis or recycling is attempted. Another is the slower coupling rate of 2-cyanoethyl *contra* methyl phosphoramidites, and the very slow coupling rate of 2-chlorophenyl phosphoramidites.

The relative rates found, however, should be used with caution under conditions where phosphoramidites and tetrazole are premixed, *i.e.* the conditions normally used for DNA synthesis. Coupling rates under such conditions are, according to the mechanism of Scheme 2, a function of the amount of phosphorotetrazolide 4 formed before the mixture enters the column, and of the reactivity of the phosphorotetrazolide. The rates studied here are probably the rates of formation of 4, which only indirectly are of use for predicitons of the coupling rates in DNA synthesis.

EXPERIMENTAL SECTION

Acetonitrile (Rathburn, HPLC grade) was dried over molecular sieves 3 Å. Chloroform and THF were freed from acids and dried by filtration through basic alumina (Woelm B, Super 1). Triethylamine was dried by filtration through basic alumina and stored over KOH pellets. Dichloromethane and EtOAc were distilled twice. Pyridine was distilled and dried over molecular sieves, 4 Å. All solvents had a water content of less than 20 $\mu g/ml^{-1}$ (CH_3CN less than 7 $\mu g/ml^{-1}$) as determined by Karl Fischer titration (Metrom

652 KF coulometer). Tetrazole was purified by sublimation at 115°C and 0.2 mmHg. Septum bottles were dried over Sicacide (Merck, art. 719) at 15 mmHg for at least one week. Silica gel for column chromatography was Merck Kiselgel 60, art. 9385.

^{1}H NMR and ^{31}P NMR spectra were obtained on a JEOL FX 90Q spectrometer; ^{1}H NMR at 89.5 MHz, chemical shifts (δ_H) relative to tetramethylsilane; ^{31}P NMR at 36.3 MHz, chemical shifts (δ_P) positive in the low field direction, external standard 85% H_3PO_4. UV absorbance (498 nm) was measured on a Visible Spectrophotometer (Philips PYE UNICAM SP6-250).

5'-O-(Dimethoxytrityl)thymidine (DMTdT),[28] 5'-O-(dimethoxytrityl)-6-N-benzoyl-2'-deoxyadenosine (DMTdA^{bz}), 5'-O-(dimethoxytrityl)thymidine-3'-yl methyl N,N-diisopropylphosphoramidite (1b),[4] 5'-O-(dimethoxytrityl)thymidine-3'-yl methyl phosphoromorpholidite (1c),[5] 5'-O-(dimethoxytrityl)thymidine-3'-yl 2-cyanoethyl N,N-diisopropylphosphoramidite (1e),[6] chlorobis(diisopropylamino)phosphine,[29] 3-hydrobutyronitrile,[30] and dichloro(2-cyano-1,1-dimethylethoxy)phosphine[9] were prepared by published methods. The phosphoramidites 1b, 1c and 1e were purified by column chromatography in the same way as 1a (see below).

3'-O-(t-Butyldiphenylsilyl)-6-N-benzoyl-2'-deoxyadenosine (2a). DMTdA^{bz} (6.5 g, 10 mmol) and imidazole (3.3 g, 48 mmol) were dried by coevaporation with dry CH_3CN (40 ml), and then dissolved in dry DMF (70 ml). To this solution was added dropwise with stirring t-butyldiphenylsilyl chloride (6.0 ml, 23 mmol) over 15 min. After stirring for 24 h at room temperature the solution was poured into 5% aq. $NaHCO_3$ (500 ml), the product extracted with CH_2Cl_2 (4 x 200 ml), and the CH_2Cl_2 phase dried over Na_2SO_4 and evaporated to an oil. The oil was dissolved in CH_3NO_2/CH_3OH (95/5, v/v, 70 ml) and $ZnBr_2$ (17 g, 75.5 mmol) added. After stirring for 1 h the solution was poured into 5% aq. NH_4HCO_3 (300 ml), extracted with CH_2Cl_2 (3 x 100 ml), and the CH_2Cl_2 phase dried over Na_2SO_4 and evaporated to an oil. The oil was dissolved in CH_2Cl_2/CH_3COCH_3 (85/15, v/v, 15 ml), and applicated on a silica gel column (diameter 7.5 cm, height 10 cm). The product was eluted with CH_2Cl_2/CH_3COCH_3 (85/15, v/v), and the appropriate fractions evaporated to a foam, which was crystallized from CH_3CN (45 ml). Yield 4.1 g (71%), mp. 164.5-165.5°C. Elemen-

tal analysis: $C_{33}H_{35}N_5O_4Si$, calc.: C 66.59%, H 5.95%, N 11.79%. Found: C 66.59%, H 5.95%, N 11.44%. δ_H ($CDCl_3$): 9.51 (s, 1H, NH), 8.63 (s, 1H, H(2)), 8.07 (s, 1H, H(8)), 8.05-7.27 (m, 15H, aromatic), 6.43 (t, 1H, H(1')), 5.60 (d, 1H, H(3')), 4.72 (s, 1H, OH), 3.78-3.20 (m, 4H, H(5') + H(2')), 1.12 (s, 9H, Bu^t).

Chloro(diethylamino)methoxyphosphine was prepared analogously to chloromethoxymorpholinophosphine[31] in 62% yield, b.p. 68-70°C at 13 mmHg, $\delta_P(CDCl_3)$ 178.2.

Chloro(N,N-methylphenylamino)methoxyphosphine. To a solution of dichloromethoxyphosphine (2.66 g, 20 mmol) and dry Et_3N (29 ml, 20 mmol) in dry ether (30 ml) was added dropwise with stirring at 25°C N-methylaniline (2.14 g, 20 mmol). After 1 h the precipitate was removed by filtration (under N_2) and washed with dry ether (2 x 10 ml). The combined solutions were evaporated *in vacuo* and the oily residue vacuum distilled. Yield 1.20 g (30%), b.p. 72-73°C at 0.3 mmHg, $\delta_P(CDCl_3)$ 173.0.

Dichloro(2-cyano-1-methylethoxy)phosphine was prepared from 3-hydroxybutyronitrile and phosphorus trichloride by the general method of Claesen *et al.*[32] in 99% yield, $\delta_P(CDCl_3)$ 176.2.

5'-O-(Dimethoxytrityl)thymidine-3'-yl methyl N,N-diethylphosphoramidite (1a). DMTdT (1.09 g, 2 mmol) was dried by coevaporation with dry CH_3CN (10 ml) and dissolved in a mixture of dry THF (6 ml) and dry Et_3N (1.2 ml). To this solution was added dropwise with stirring chloro(diethylamino)methoxyphosphine (0.67 g, 3.5 mmol). After 30 min at room temperature the suspension was filtered, and the precipitate washed with dry THF (2x2 ml). The combined THF solutions were poured into a mixture of EtOAc (80 ml) and sat. aq. $NaHCO_3$ (20 ml), the organic phase was separated and extracted with sat. aq. $NaHCO_3$ (2 x 20 ml), and then dried over $MgSO_4$ and the solvent removed *in vacuo*. The residue was dissolved in a mixture of CH_2Cl_2, EtOAc, and Et_3N (45/45/10 v/v, 3 ml), applicated on a silica gel column (diam. 4 cm, height 7 cm) and eluted with the same solvent mixture. The fractions containing the product (TLC) were pooled and evaporated, and the residue dissolved in dry toluene (8 ml) and precipitated into dry hexane (200 ml) at 0°C. After lyophilisation from dry CH_3CN and drying *in vacuo* 1a was obtained as a white powder, 0.76 g (56% yield), $\delta_P(CDCl_3)$ 149.2, 148.5.

5'-O-(Dimethoxytrityl)thymidine-3'-yl methyl N,N-methylphenyl-phosphoramidite (1d) was prepared from DMTdT and chloro(N,N-methyl phenylamino)methoxyphosphine in the same way as 1a. Yield 0.94 g (66%), $\delta_P(CDCl_3)$ 143.5, 143.2.

5'-O-(Dimethoxytrityl)thymidine-3'-yl 2-cyano-1-methylethyl N,N-diisopropylphosphoramidite (1f). DMTdT (0.54 g, 1 mmol) was dried by coevaporation with dry CH_3CN (5 ml) and dissolved in dry THF/pyridine (2/1 v/v, 3 ml). This solution was added with stirring during 15 min to a solution of dichloro(2-cyano-1-methylethoxy)phosphine (0.56 g, 3 mmol) in THF/pyridine (2/1 v/v, 3 ml) at -78°C. After 30 min at room temperature the mixture was cooled on ice and dry diisopropylamine (1.0 ml, 7 mmol) added dropwise. The suspension was stirred for 15 min and then poured into a mixture of EtOAc (100 ml) and 10% aq. $NaHCO_3$ (50 ml). The organic layer was washed with 10% aq. $NaHCO_3$ (2x50 ml), dried over Na_2SO_4 and evaporated. The residue was dissolved in EtOAc (5 ml) containing Et_3N (2 drops) and applicated on a silica gel column (diameter 4 cm, height 10 cm). The product was eluted with a mixture of hexane, CH_3COCH_3 and Et_3N (60/30/10 v/v), and the appropriate fractions evaporated. The product was coevaporated with dry CH_3CN (2x5 ml) to a white foam. Yield 0.14 g (10%), $\delta_P(CDCl_3)$ 147.4, 147.0, 146.9 (four diastereoisomers expected).

5'-O-(Dimethoxytrityl)thymidine-3'-yl 2-cyano-1,1-dimethylethyl N,N-diisopropylphosphoramidite (1g) was prepared from DMTdT and dichloro(2-cyano-1,1-dimethylethoxy)phosphine in the same way as 1f. Yield 0.20 g (27%), $\delta_P(CDCl_3)$ 139.3.

5'-O-(Dimethoxytrityl)thymidine-3'-yl 2-chlorophenyl N,N-diisopropylphosphoramidite (1h). DMTdT (0.54 g, 1 mmol) was dried by coevaporation with dry dioxane (5 ml), dissolved in dioxane (5 ml), and dry Et_3N (0.21 ml, 1.5 mmol) added, followed by chloro-bis(diisopropylamino)phosphine (0.31 g, 1.2 mmol). After stirring for 15 min the suspension was filtered, and 2-chlorophenol (0.19 g, 1.5 mmol) and tetrazole (0.07 g, 1 mmol) added. The mixture was stirred overnight, the solvent evaporated, and the residue dissolved in EtOAc. Washing with aq. $NaHCO_3$, purification by column chromatography, and precipitation was performed as described for 1a. Yield 0.51 g (62%), $\delta_P(CDCl_3)$ 147.5, 147.1.

Kinetic experiments. A dried 5 ml septum bottle containing a

teflon magnetic stirrer was charged with 1 (0.15 mmol), 2a (0.075 mmol) and dry CH_3CN (4.25 ml). A slight nitrogen pressure was applied *via* a needle through the septum, and the solution was stirred for 30 min in an ice/water bath. At time zero a 0^oC solution of tetrazole (0.2 M in CH_3CN, 0.75 ml) was added. At specific time intervals was 0.10 ml reaction mixture withdrawn with a 1 ml plastic syringe and quenched by injection into a mixture of CH_2Cl_2, EtOAc, and Et_3N (45/45/10 v/v, 0.8 ml). After completion of the kinetic run an aliquot of each sample was transferred onto a TLC plate (Merck kiesel gel 60, Art. 5554, 20x20 cm) which was evolved in a mixture of CH_2Cl_2, EtOAc, and Et_3N (45/45/10 v/v). The dried plate was sprayed with 20% H_2SO_4 in MeOH, and the red spots corresponding to 1, 3 and hydrolysed 1 were separately scratched off for each sample and eluted with 0.10 M p-toluenesulfonic acid in CH_3CN (1.00 ml). The absorbance at 498 nm, A_3, was measured directly on these solutions in a 1 mm cuvette, and [3] calculated from the formula

$$[3] = \frac{A_3}{A_1 + A_3 + A_{hy}} \times 0.030\ M$$

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